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### **Research Article**

Abnormal proliferation of vascular smooth muscle cells (VSMCs) contributes to intimal hyperplasia during atherosclerosis and restenosis, but the endogenous cell cycle regulatory factors underlying VSMC growth in response to arterial injury are not well understood. In the present study, we report that downregulation of cyclin-dependent kinase 2 (cdk2) activity in serum-deprived VSMCs was associated with the formation of complexes between cdk2 and its inhibitory protein p27(KIP1) (p27). Ectopic overexpression of p27 in serum-stimulated VSMCs resulted in the inhibition of cdk2 activity and repression of cyclin A promoter activity. Collectively, these findings indicate that p27 may contribute to VSMC growth arrest in vitro. Using the rat carotid model of balloon angioplasty, a marked upregulation of p27 was observed in injured arteries. High levels of p27 expression in the media and neointima correlated with downregulation of cdk2 activity at 2 wk after angioplasty, and adenovirus-mediated overexpression of p27 in balloon-injured arteries attenuated neointimal lesion formation. Thus, the inhibition of cdk2 function and repression of cyclin A gene transcription through the induction of the endogenous p27 protein provides a mechanism for the inhibition of VSMC growth at late time points after angioplasty.

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## Downregulation of Cyclin-dependent Kinase 2 Activity and Cyclin A Promoter Activity in Vascular Smooth Muscle Cells by p27<sup>KIP1</sup>, an Inhibitor of Neointima Formation in the Rat Carotid Artery

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### Abstract

Abnormal proliferation of vascular smooth muscle cells (VSMCs) contributes to intimal hyperplasia during atherosclerosis and restenosis, but the endogenous cell cycle regulatory factors underlying VSMC growth in response to arterial injury are not well understood. In the present study, we report that downregulation of cyclin-dependent kinase 2 (cdk2) activity in serum-deprived VSMCs was associated with the formation of complexes between cdk2 and its inhibitory protein p27<sup>KIP1</sup> (p27). Ectopic overexpression of p27 in serum-stimulated VSMCs resulted in the inhibition of cdk2 activity and repression of cyclin A promoter activity. Collectively, these findings indicate that p27 may contribute to VSMC growth arrest in vitro. Using the rat carotid model of balloon angioplasty, a marked upregulation of p27 was observed in injured arteries. High levels of p27 expression in the media and neointima correlated with downregulation of cdk2 activity at 2 wk after angioplasty, and adenovirus-mediated overexpression of p27 in balloon-injured arteries attenuated neointimal lesion formation. Thus, the inhibition of cdk2 function and repression of cyclin A gene transcription through the induction of the endogenous p27 protein provides a mechanism for the inhibition of VSMC growth at late time points after angioplasty. (*J. Clin. Invest.* 1997; 99: 2334–2341.) Key words: restenosis • cell cycle control • p27<sup>KIP1</sup> • adenovirus • vascular smooth muscle cells

### Introduction

Atherosclerosis and restenosis are multifactorial processes which result from a complex cascade of events involving abnormal proliferation of vascular smooth muscle cells (VSMCs)<sup>1</sup> (1–5). Although the occurrence of restenosis in ~ 20–55% of patients after clinically successful revasculariza-

tion remains the major limitation to percutaneous transluminal coronary angioplasty (6–11), no clinical trials have yet demonstrated convincing effects in reducing the incidence of restenosis (12–14). Previous studies using several models of angioplasty have demonstrated a rapid proliferative response of VSMCs in the media, followed by a second peak of proliferation in the neointima, which then declines to basal levels by 2–4 wk after angioplasty (15–18). While the contribution of several cell cycle control genes and growth stimulatory genes to VSMC growth has been well established (19–28), the endogenous factors underlying the reestablishment of the quiescent phenotype in VSMCs after angioplasty are largely unknown.

Cellular proliferation is controlled by multiple holoenzymes comprising a catalytic cyclin-dependent protein kinase (cdk) and a cyclin regulatory subunit (29–33). Functional cdk/cyclin holoenzymes phosphorylate target protein substrates that facilitate progression through the cell cycle. cdk activity is negatively regulated by the interaction with specific cdk inhibitory proteins that cause cell cycle arrest in G<sub>1</sub> phase when overexpressed in transformed and nontransformed cell lines (34–39). Mice lacking the cdk inhibitor function of p27 display enhanced growth and multiple organ hyperplasia (40–42). In addition to its role in the control of cell proliferation during normal development, p27 appears to function as a tumor suppressor (43), and reduced expression of p27 has been associated with poor cancer patient survival (44–46). In the present study, we provide evidence suggesting the role of p27 as a negative regulator of VSMC growth in vitro and after angioplasty in vivo.

### Methods

**Cell culture, adenovirus infection, and transient transfection assays.** Cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> 95% O<sub>2</sub> atmosphere in media supplemented with 2 mM L-glutamine, 200 U/ml penicillin, 0.25 mg/ml streptomycin, and serum as indicated. Primary rat aorta VSMCs were isolated essentially as described by Pickering et al. (47) and maintained in DME supplemented with 10% FCS (growth medium). To render cells quiescent, primary cultures were maintained for 3 d in DME supplemented with 0.5% FCS. Rat pulmo-

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1. **Abbreviations used in this paper:** Adp27, recombinant, replication-defective adenovirus containing human p27<sup>KIP1</sup> cDNA expression cassette; Adβgal, recombinant, replication-defective adenovirus containing β-galactosidase cDNA expression cassette; cdk, cyclin-dependent kinase; VSMC, vascular smooth muscle cell; Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

nary arterial smooth muscle cells (PAC1) (48) were maintained in medium 199 supplemented with 20% FCS.

Replication-defective adenoviruses directing expression of human p27 (Adp27) and  $\beta$ -galactosidase (Ad $\beta$ gal) under the transcriptional control of the cytomegalovirus promoter have been described previously (43). Primary cultures of rat aorta VSMCs were cultured in growth medium to  $\sim$ 60% confluence, and were incubated with the indicated amounts of adenovirus for 24 h. Cells were then washed with PBS and fed fresh growth medium. After an additional 16 h of incubation, cells were harvested for the preparation of whole cell extracts for p27 immunoblot analysis and anti-cdk2 kinase assays (see below).

PAC1 cells were transiently transfected using Lipofectamine reagent as recommended by the manufacturer (GIBCO Laboratories, Grand Island, NY). Reporter vectors containing the -356 to +109 c-fos and -79 to +100 cyclin A gene 5' flanking sequence upstream from a promoterless luciferase cDNA, and the expression vector pAC-CMV-p27, were provided by M. Simonson (Case Western University, Cleveland, OH), J. Sobczak-Thopot (INSERM, Paris), and J. Massagué (Memorial Sloan-Kettering Cancer Center, New York), respectively. Transfection mixtures contained 36  $\mu$ g of Lipofectamine, 5  $\mu$ g of reporter vector, 0–1  $\mu$ g of pAC-CMV-p27, and empty pAC-CMV plasmid to equalize the total amount of DNA. Differences in transfection efficiency were corrected relative to the level of alkaline phosphatase activity produced from cotransfected pSVA-PAP plasmid (0.5  $\mu$ g per transfection), which contains the reporter gene under the control of the SV40 promoter/enhancer (49). Transfected cells were maintained in medium 199 supplemented with 20% FCS for 2 d, and then whole cell extracts were prepared to measure luciferase and alkaline phosphatase activity as previously described (50).

**Balloon injury in the rat carotid artery.** Balloon denudation of the left common carotid artery in male Sprague-Dawley rats weighing 400–500 g was performed essentially as described by Clowes et al. (16). Briefly, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital solution (45 mg/kg), the bifurcation of the left common carotid artery was surgically exposed, and the left common and internal carotid arteries were temporarily ligated. A 2F embolectomy catheter (Baxter Healthcare Corp., McGaw Park, IL) was then introduced into the external carotid, and advanced to the distal ligation of the common carotid. The balloon was inflated with saline and drawn towards the arteriotomy site three times to denude the artery. After withdrawing the angioplasty catheter, the proximal external carotid artery was permanently ligated, and blood flow was restored to the common carotid artery by release of the ligatures. At different time points after angioplasty, arteries were perfused in situ with saline through a cannula inserted into the left ventricle, dissected free of the surrounding tissue, snap-frozen, and stored at  $-80^{\circ}\text{C}$  until the preparation of cell extracts (see below).

**Preparation of cell extracts, Western blot analysis, and cdk2 kinase assay.** Whole cell extracts from rat carotid arteries and cultured cells were prepared in ice-cold lysis buffer (50 mM Hepes [pH 7.6], 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 10 mM  $\beta$ -glycerophosphate, 0.1 mM  $\text{NaVO}_3$ , 1 mM NaF, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 1 mM PMSF, and 4  $\mu$ g/ml leupeptin). Arterial extracts were prepared using a homogenizer (Tissumizer Mark II; Tekmar Co., Cincinnati, OH). Lysates were centrifuged at  $4^{\circ}\text{C}$  for 10 min in a microfuge set at maximum speed, and the supernatant was stored at  $-80^{\circ}\text{C}$  in small aliquots. The following dilutions of primary antibodies were used for Western blot analysis: anti-p27 sc-528 (1:250), anti-cdk2 sc-163 (1:500), and anti-cyclin E sc-481 (1:250) (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were detected using an ECL detection kit according to the recommendations of the manufacturer (Amersham Corp., Arlington Heights, IL). For cdk2-dependent kinase assays, cell lysates (50–75  $\mu$ g protein) were incubated at  $4^{\circ}\text{C}$  for 1.5–2 h under constant rotation in 0.5 ml of lysis buffer containing 0.15  $\mu$ g of anti-cdk2 antibodies and 25  $\mu$ l of protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology). Immunocomplexes were washed three times with lysis buffer and twice with ki-

nase buffer (40 mM Tris-Cl [pH 7.6], 20 mM  $\text{MgCl}_2$ , 2 mM DTT). Subsequently, the beads were resuspended in 30  $\mu$ l of kinase buffer containing 2  $\mu$ g of histone H1 (Boehringer Mannheim Biochemicals, Indianapolis, IN), 7  $\mu$ M ATP, and 5  $\mu$ Ci of [ $\gamma$ - $^{32}\text{P}$ ]ATP. The reaction mixtures were incubated at  $30^{\circ}\text{C}$  for 30 min and then separated on 12% SDS/polyacrylamide gels. Gels were stained with Coomassie Blue (Sigma Chemical Corp., St. Louis, MO), dried, and autoradiographed. Quantification of the signal was performed by counting individual histone H1 bands in a scintillation counter. Background counts per minute determined from regions of the dried gel that did not contain protein were subtracted.

**Immunohistochemistry.** Rat carotid arteries were fixed by immersion in 100% methanol overnight, embedded in paraffin, and cut in 5- $\mu$ m sections for immunohistochemistry using a streptavidin-peroxidase detection system according to the recommendations of the manufacturer (Signet Laboratories Inc., Dedham, MA). Specimens were incubated overnight at  $4^{\circ}\text{C}$  with anti-p27 mouse mAb (1:20 dilution) (Oncogene Research Products, Cambridge, MA). p27 immunocomplexes were detected with AEC substrate (BioGenex Labs., San Ramon, CA). Specimens were mounted with glycerol gelatin aqueous mounting media (Sigma Chemical Co.) and examined on a microscope (Vanox-T; Olympus Optical Co., Shinjuku, Japan). Pictures were recorded on Kodak Gold Plus film (Eastman Kodak Co., Rochester, NY).

**Localized arterial infection with adenovirus vectors and quantification of intimal hyperplasia.** Balloon injury in the rat carotid artery was carried out as described above. After withdrawing the angioplasty catheter, Adp27 or Ad $\beta$ gal was injected via a cannula inserted just proximal to the carotid bifurcation. Viral infusion mixtures contained 10  $\mu$ l of adenovirus vector (total  $0.5 \times 10^9$  plaque-forming units) and 90  $\mu$ l of 15% (wt/vol) Poloxamer 407 (BASF Corp., Parsippany, NJ). This pluronic gel has been shown to enhance adenovirus infectivity in VSMC in vitro and after angioplasty in the rat carotid artery (51, 52). The adenovirus solution was incubated for 20 min, after which the viral infusion was withdrawn, the cannula was removed, and blood flow was restored. At 12 d after angioplasty, rats were anesthetized and arteries were perfused in situ with saline followed by 2% paraformaldehyde (in PBS) at a pressure of 90 mmHg through a cannula inserted into the left ventricle. Tissues were immersed in 2% paraformaldehyde overnight, and were then embedded in paraffin for tissue sectioning (see below). Cross sections (5- $\mu$ m) were stained with elastic-trichrome stain, and histological images were projected onto a digitizing board (Summagraphics Corp., Fairfield, CT). Planimetric analysis of the projected sections was performed by a single operator who was blinded to treatment group. A computerized sketching program (MacMeasure Version 1.9; National Institute of Mental Health, Bethesda, MD) was used to determine the medial, intimal, and luminal areas. For each artery, two sections from the middle third and two sections from one proximal third of the injured segment (taken at least 100  $\mu$ m apart) were analyzed. The four measurements from each artery were averaged to determine the extent of intimal lesion. Results were expressed as mean  $\pm$  SEM. Differences between Adp27- and Ad $\beta$ gal-infected arteries were analyzed using an unpaired two-tailed Student's *t* test.

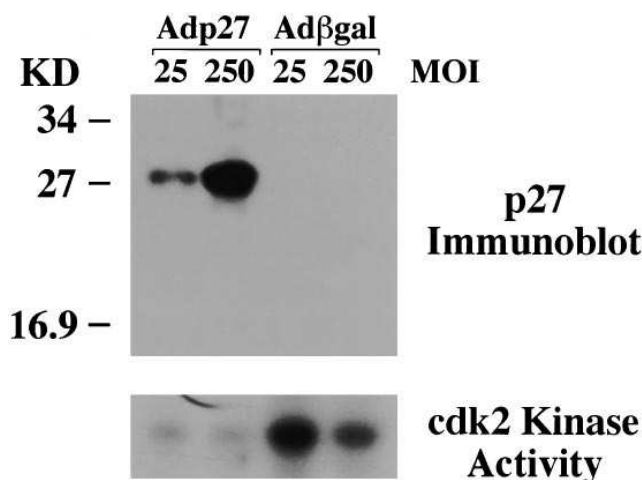
Recombinant p27 and  $\beta$ -galactosidase expression in injured arteries was evaluated 3 d after angioplasty and adenovirus infection. After perfusion with saline, two arteries were snap-frozen to examine p27 expression by Western blot analysis (see above), and four arteries were processed for evaluation of  $\beta$ -galactosidase activity by histochemical staining with the chromogenic substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (Xgal). To obviate the issue of endogenous  $\beta$ -galactosidase activity (53), a modified adenovirus vector containing nuclear localized  $\beta$ -galactosidase cDNA expression cassette under the control of the cytomegalovirus promoter was used for these studies (courtesy of Didier Branellec, Rhône-Poulenc Rorer, Vitry, France). Arteries for Xgal staining were fixed for 15 min by immersion in 1% paraformaldehyde, rinsed with PBS and then incubated overnight at  $37^{\circ}\text{C}$  in PBS, containing 5 mM  $\text{K}_4\text{Fe}(\text{CN})_6$ , 5 mM

$K_3Fe(CN)_6$ , 2 mM  $MgCl_2$ , 0.02% Nonidet P-40, 0.01% sodium deoxycholate, and 1 mg/ml Xgal. Stained arteries were paraffin-embedded, cut into 5- $\mu$ m sections, mounted in microscope slides and counterstained with hematoxylin and eosin.

## Results

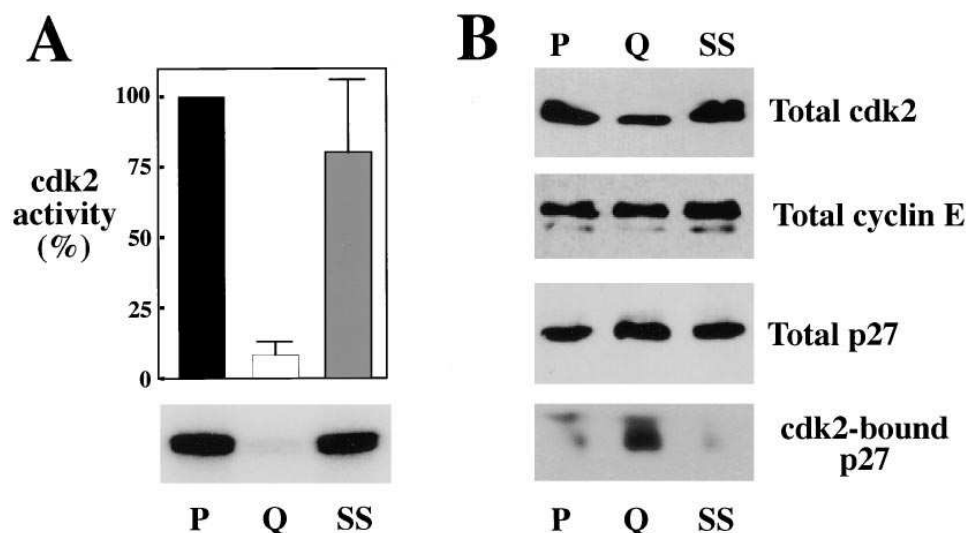
**Inhibition of cdk2 kinase activity by p27 in primary cultures of VSMCs.** It has been demonstrated previously that cdk2 function is required for intimal smooth muscle cell accumulation after angioplasty in the rat carotid artery (20, 23), and cdk2 expression correlates with VSMC proliferation after angioplasty in both human and rat arteries (54). Therefore, the present study aimed to investigate the mechanisms underlying the regulation of cdk2 activity in VSMCs. As shown in Fig. 1 A, cdk2 activity in primary cultures of VSMCs was markedly downregulated in serum-deprived cells (*Q*, quiescent), as compared to asynchronously growing cells (*P*, proliferating), and quiescent cells that had been serum-stimulated for 1 d (*SS*, serum-stimulated). Abundant expression of both cdk2 and its regulatory subunit cyclin E in serum-deprived VSMCs (Fig. 1 B) suggested that the downregulation of cdk2 activity in these cells was achieved posttranslationally. Thus, we examined the pattern of expression of the cdk2 inhibitory protein p27 in cultured VSMCs. While the total pool of p27 remained nearly unchanged under all experimental conditions tested, p27 was preferentially detected in anti-cdk2 immunoprecipitates harvested from serum-deprived VSMCs (Fig. 1 B). In marked contrast, active cdk2 complexes in proliferating and serum-stimulated VSMCs did not contain detectable levels of p27 (Fig. 1 B). These results suggest that p27 contributes to the inhibition of cdk2 activity in serum-deprived VSMCs.

Direct evidence for inhibition of cdk2 activity in VSMCs by p27 was provided using an adenovirus vector system directing the expression of p27 (Adp27). We demonstrated previously that infection of tumor cells with Adp27 is associated with a marked decrease in cdk2 kinase activity, inhibition of [ $^3H$ ]thymidine incorporation, and cell cycle arrest in  $G_1$  (43). Overex-



**Figure 2.** Adenovirus-mediated overexpression of p27 inhibits cdk2 kinase activity in VSMCs. Primary cultures of rat aorta VSMCs were grown to ~60% confluence, and then infected with the indicated amounts of adenovirus directing the expression of p27 (Adp27) and  $\beta$ -galactosidase (Ad $\beta$ gal). After 40 h, cells were harvested for the preparation of whole-cell extracts. Expression of p27 was analyzed by Western blot. Note that the endogenous p27 is not detected under these conditions (15  $\mu$ g of protein extract, 1-min exposure), as compared to the analysis in Fig. 1 (75  $\mu$ g of protein extract, 15-min exposure). For cdk2 kinase assay, 50  $\mu$ g of protein extract was immunoprecipitated with anti-cdk2 antibodies.

pression of p27 in VSMCs infected with Adp27 was demonstrated by Western blot analysis, as compared to cells infected with a control adenovirus encoding  $\beta$ -galactosidase (Ad $\beta$ gal) (Fig. 2). Biological activity of overexpressed p27 protein was demonstrated in anti-cdk2 immunoprecipitates, which revealed low levels of cdk2-dependent kinase activity in Adp27-infected cells (Fig. 2). Thus, these findings suggest that inhibi-



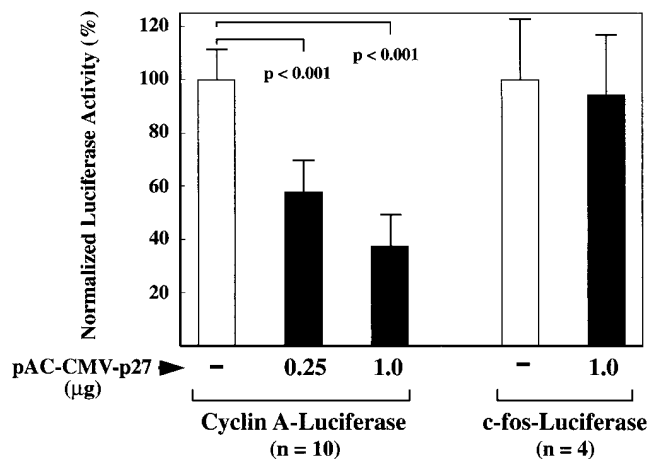
**Figure 1.** Cdk2 inactive complexes in serum-deprived VSMCs contain the inhibitory protein p27. Primary cultures of rat aorta VSMCs were maintained at subconfluent densities in 10% FCS (*P*, proliferating), at confluent densities for 3 d in 0.5% FCS (*Q*, quiescent), or serum stimulated for 1 d with 10% FCS after 3 d of serum deprivation (*SS*, serum-stimulated). (A) Histone H1 kinase activity was assayed in anti-cdk2 immunoprecipitates prepared from cell lysates (100- $\mu$ g protein extract). Bars represent the mean  $\pm$  SEM of three independent assays. A representative autoradiograph is shown. (B) Western blot analysis was performed to determine the total amount of cdk2, cyclin E, and p27 (75- $\mu$ g protein extract). Analysis

was performed three to four times using two different batches of lysates. Representative blots are shown. To investigate the association between cdk2 and p27, 0.75 mg of protein extract was first immunoprecipitated with anti-cdk2 antibodies, and then the immunoprecipitate was examined by Western blot analysis using p27 antibodies.

tion of cdk2 kinase activity by p27 contributes to VSMC growth arrest in vitro.

**p27 represses cyclin A promoter activity in VSMCs.** To test whether p27 may limit VSMC growth through transcriptional repression of positive regulators of cell cycle progression, we next examined the effect of p27 on cyclin A promoter activity in PAC1 cells. It has been demonstrated previously that cell cycle regulation of the cyclin A gene promoter in NIH 3T3 fibroblasts is mediated by sequences extending from -79 to +100 relative to the predominant transcription start site (55), and binding of E2F to the -37 to -33 sequence is required for this regulation (56). In agreement with these findings, transient transfection experiments demonstrated a marked downregulation of the -79 to +100 cyclin A promoter in serum-deprived PAC1 cells relative to cells maintained in 20% FCS (data not shown). Serum-dependent transactivation of the -79 to +100 cyclin A promoter fragment was inhibited by p27 in a dose-dependent manner (Fig. 3). In marked contrast, p27 did not affect activity from the -356 to +109 c-fos promoter, a well-characterized serum-dependent gene that has not been reported to be E2F responsive. Similar results were obtained in 10T1/2 fibroblasts (data not shown).

**Induction of p27 protein expression after angioplasty in the rat carotid artery.** The results thus far suggest that inhibition of cdk2 kinase activity and transcriptional repression of cyclin A gene expression contributes to VSMC growth arrest in vitro. To test whether p27 may limit VSMC growth in vivo, we examined its pattern of expression after angioplasty in the rat carotid artery. Western blot analysis disclosed a marked induc-



**Figure 3.** p27 represses cyclin A promoter activity in VSMCs. PAC1 cells were cotransfected with 5 µg of reporter vector (cyclin A or c-fos promoters cloned upstream of the promoterless luciferase cDNA), and with 0–1 µg of the expression vector pAC-CMV containing the p27 cDNA. Transfection mixes also contained empty pAC-CMV vector to equalize the total amount of transfected DNA, and 0.5 µg of the alkaline phosphatase expression vector pSVAPAP. Cells were maintained in 20% FCS for 2 d, lysed, and assayed for luciferase activity, which was normalized relative to the alkaline phosphatase activity from the cotransfected internal control pSVAPAP vector. Normalized activity in the absence of p27 expression vector is defined as 100%. Values represent the mean ± SEM of the indicated number of independent experiments (n). Statistically significant differences relative to control are indicated (Student's *t* test) (see Methods for details).

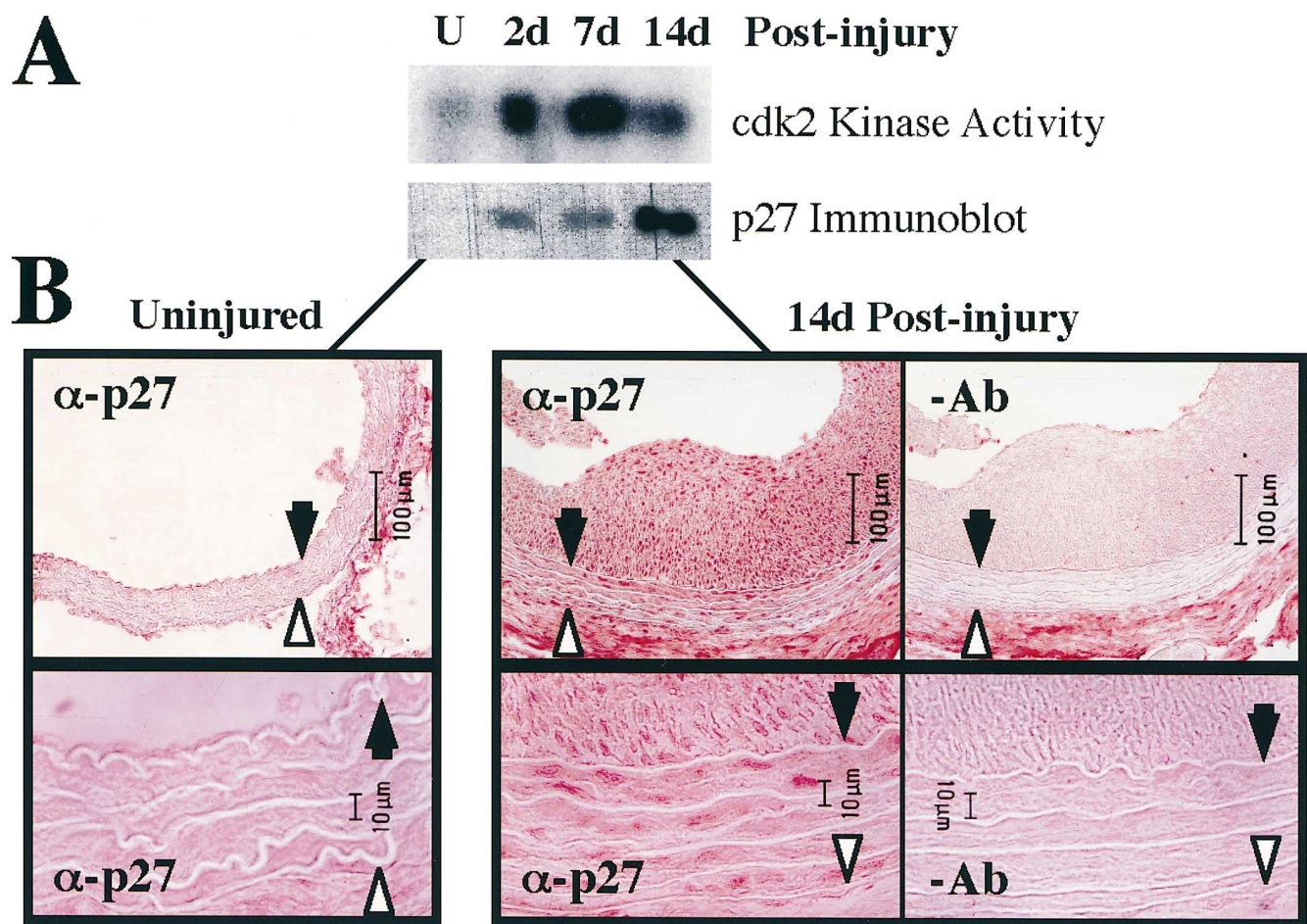
tion of p27 after balloon angioplasty (Fig. 4 A). Notably, high levels of p27 correlated with downregulation of cdk2-associated kinase activity at 2 wk after injury (Fig. 4 A), coincident with the termination of proliferation in this animal model of vascular injury (16, 57). To examine the spatial pattern of expression of p27 after angioplasty in the rat carotid artery, we performed immunohistochemical analysis. As compared to uninjured arteries, p27 was abundantly expressed within the media and neointima at 2 wk after injury (Fig. 4 B). At this time point, expression of the phenotypic marker smooth muscle α-actin was detected throughout the media and neointima (data not shown). Taken together, these results suggested that induction of p27 may contribute to the reestablishment of the postmitotic state of VSMCs after angioplasty in the rat carotid artery.

**Localized arterial infection with Adp27 inhibits intimal hyperplasia after angioplasty in the rat carotid artery.** To test directly whether p27 could inhibit neointima formation, we delivered Adp27 into the vessel wall of rat carotid arteries immediately after balloon angioplasty. Injured arteries were also treated with Adβgal control vector. Arteries were harvested 3 d after adenoviral infection to examine expression of recombinant p27 and β-galactosidase. The pooled tissue from two arteries was used for Western blot analysis, which disclosed higher levels of p27 in arteries infected with Adp27 as compared to endogenous p27 in the Adβgal-infected group (Fig. 5 A). Evidence of β-galactosidase activity was evaluated using Xgal histochemical staining and an adenovirus vector modified to include a nuclear localization sequence. Gross examination disclosed β-galactosidase activity over the entire length of three out of four Adβgal-infected arteries (data not shown). Microscopic examination of cross sections revealed dark blue staining in the media of Adβgal-infected arteries which was more intense in the nucleus (Fig. 5 B). Xgal staining was seen not only in superficial but also in deeper cell layers of the media in Adβgal-infected arteries. Distribution of transfected cells in cross sections was heterogenous, with positive regions alternating with negative areas over the entire length of the injured arterial segment. Analysis of three Adp27-infected arteries disclosed undetectable β-galactosidase activity (Fig. 5 B). Collectively, these data demonstrated successful adenovirus-mediated overexpression of recombinant p27 and β-galactosidase after angioplasty in the rat carotid artery.

In a second set of experiments, rats were killed 12 d after adenoviral infection to perform quantitative morphometric analysis on cross sections of the injured vessels. As shown in Fig. 5 C, a significant reduction of 49% in the intimal to medial area ratios was observed in Adp27-infected arteries ( $0.45 \pm 0.084$ ), as compared to arteries infected with Adβgal ( $0.88 \pm 0.114$ ,  $P = 0.013$  versus Adp27 group). Similarly, luminal narrowing was reduced by 50% in the Adp27-infected arteries ( $18.08 \pm 3.84\%$ ) relative to Adβgal-treated vessels ( $35.95 \pm 4.75\%$ ,  $P = 0.015$  versus Adp27 group). These results demonstrate that adenovirus-mediated overexpression of p27 after angioplasty attenuates intimal hyperplasia in the rat carotid artery.

## Discussion

Although the requirement of several positive regulators of VSMC proliferation has been well established using different



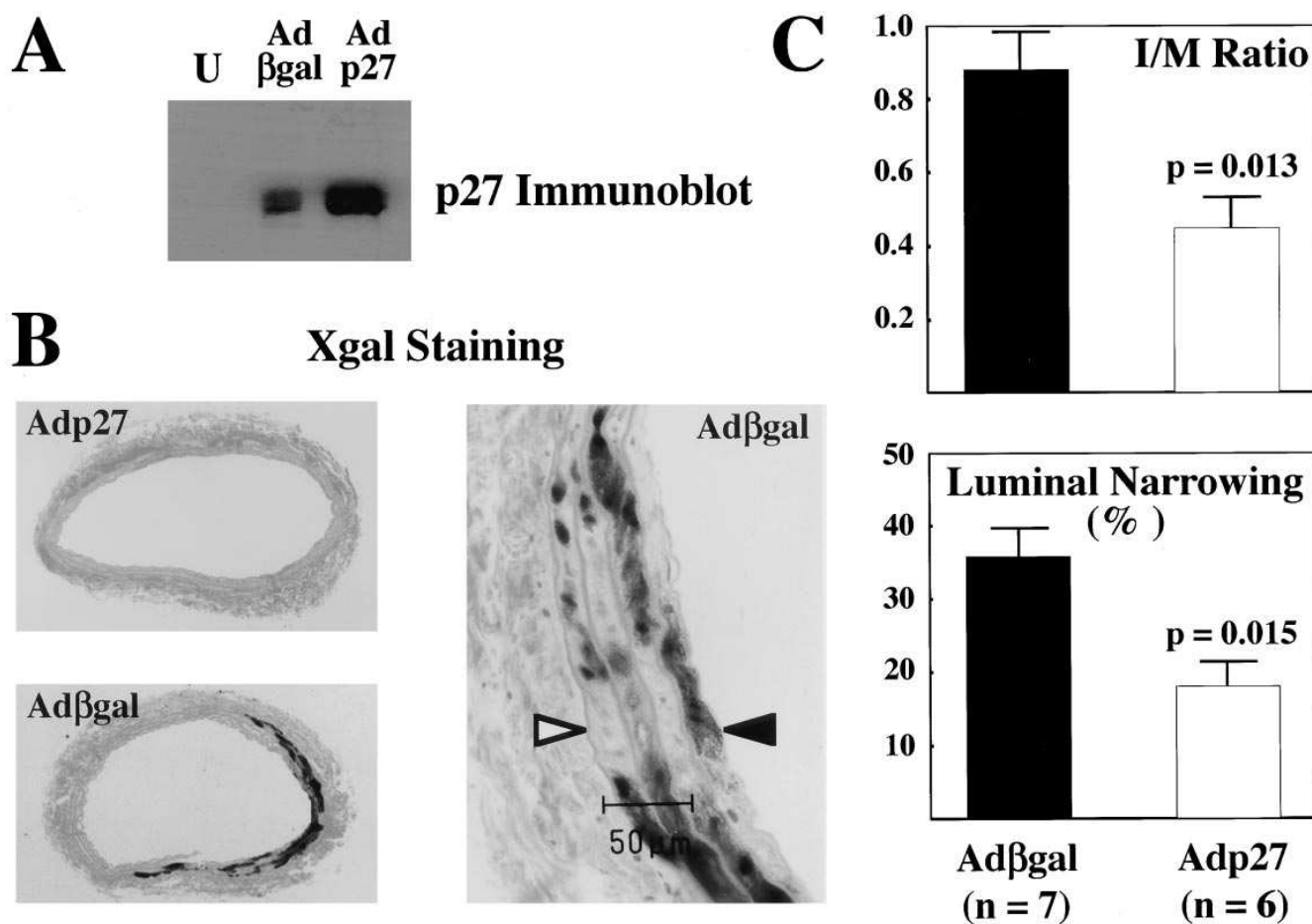
**Figure 4.** Upregulation of p27 after angioplasty in the rat carotid artery. Cell extracts from uninjured (U) and injured arteries were prepared from the pooled tissue from 8 to 10 arteries at each time point. (A) Lysates were assayed for cdk2-dependent histone H1 kinase activity (75  $\mu$ g) and p27 immunoblot (50  $\mu$ g). Note that downregulation of cdk2 activity at 14 d after injury correlated with high levels of p27. (B) Immunostaining for p27 in uninjured (left) and 14 d postinjured (right) arteries using a mouse mAb ( $\alpha$ -p27). For control, primary antibody was omitted (-Ab). Note that the signal in the adventitia is nonspecific. Controls in which the biotinylated anti-mouse IgG was omitted revealed lack of specific p27 staining as well. Black and white arrowheads point to the internal and external elastic laminae, respectively.

models of angioplasty (19–28), the molecular basis of VSMC growth arrest at late time points after angioplasty is poorly understood. The present study implicates the cdk inhibitory protein p27 as a negative regulator of VSMC growth in vitro and after angioplasty in vivo. Inactive cdk2 complexes harvested from serum-deprived VSMCs contained high levels of p27, as compared to active cdk2 complexes isolated from serum-stimulated cultures. Ectopic overexpression of p27 in VSMCs downregulated cdk2 activity and repressed transcription from the  $-79/+100$  cyclin A promoter region, which contains an E2F-binding site required for cell cycle-regulated cyclin A gene expression (56). These data are consistent with recent findings demonstrating the requirement of E2F for p27-dependent repression of the  $-7300/+11$  cyclin A promoter in NIH3T3 fibroblasts (58). Taken together with the requirement of cdk2 and cyclin A for cell cycle progression (59–61), these findings suggest the involvement of p27 as a mediator of VSMC growth arrest in vitro. In this regard, evidence has been presented recently suggesting that polymerized collagen inhibits VSMC proliferation in vitro through  $\alpha 2$  integrin-mediated

upregulation of p27 (62). It is unclear why p27 preferentially associated with cdk2 in serum-deprived VSMCs, even though the total pool of p27 was not regulated by serum in cultured VSMCs (Fig. 1 B). Others have suggested that increased association between p27 and cdk2 in MvLu mink epithelial cells may be due to the release of p27 from cdk4/cyclin D complexes (63, 64). Consistent with this notion, formation of cdk2/p27 complexes in serum-deprived VSMCs correlated with a marked downregulation of cdk4 and cyclin D1 protein levels (data not shown).

We previously demonstrated a correlation between VSMC proliferation and the expression of cdk2 and its regulatory subunits, cyclins E and A, after angioplasty in rat and human arteries (54). The present study suggests that induction of the endogenous p27 protein in the injured arterial wall may contribute to the reestablishment of the quiescent phenotype at late time points after angioplasty. Direct evidence implicating p27 as a negative regulator of VSMC proliferation after angioplasty was provided using an adenovirus delivery system. Overexpression of p27 after localized delivery of Adp27 to de-





**Figure 5.** Adenovirus-mediated overexpression of p27 after angioplasty reduces intimal lesion formation. Rat carotid arteries were incubated for 20 min with Adp27 or Adβgal immediately after balloon angioplasty. (A) Western blot analysis was performed on the pooled tissue from two arteries harvested 3 d after infection to examine the expression of p27. U, uninjured uninfected arteries. (B) Arteries were harvested 3 d after infection to examine β-galactosidase activity using Xgal substrate. Blue staining in Adβgal-infected arteries was observed in the media, which was more intense in the nucleus because of the use of a modified adenovirus vector containing nuclear localized β-galactosidase. Black and white arrowheads point to the internal and external elastic laminae, respectively. (C) Arteries were harvested 12 d after infection for morphometric analysis. The intimal to medial area ratio (I/M) was reduced by 49% in Adp27-infected arteries, as compared to arteries treated with Adβgal ( $P < 0.013$ ). Likewise, the percentage of luminal narrowing was reduced by 50% in Adp27-infected arteries ( $P < 0.015$ ).

nuded arteries reduced neointima formation by 49% relative to arteries infected with Adβgal control vector. Likewise, previous studies using adenovirus expression vectors for the growth inhibitory retinoblastoma and p21 proteins have demonstrated a ~50% reduction of intimal thickening after angioplasty in rat and porcine arteries (65–67). Conversely, human cytomegalovirus-mediated inhibition of the tumor suppressor protein p 53 may contribute to the development of restenosis (68). Collectively, these studies support the notion that deregulated function of growth suppressor molecules affects VSMC proliferation in vivo, thus suggesting novel strategies to prevent restenosis.

In summary, the present study suggests a mechanism by which suppression of cdk2 activity and transcriptional repression of cyclin A gene expression through upregulation of p27 may limit VSMC growth after angioplasty. Of note, p27- and p21-dependent suppression of cyclin A, cdc2, E2F-1, and dihydrofolate reductase promoter activity has been recently demonstrated in murine fibroblasts (58, 69, 70), and intimal cell growth termination after angioplasty in porcine arteries was

associated with the induction of endogenous p21 (67). Elucidating the signals underlying the upregulation of cdk inhibitors and, ultimately, repression of cyclin gene expression at late time points after angioplasty, should improve our understanding of the pathobiology of restenosis.

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